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Neurospora Clock-Controlled Gene 9 (*ccg-9*) Encodes Trehalose Synthase: Circadian Regulation of Stress Responses and Development

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The circadian clock of *Neurospora crassa* regulates the rhythmic expression of a number of genes encoding diverse functions which, as an ensemble, are adaptive to life in a rhythmic environment of alternating levels of light and dark, warmth and coolness, and dryness and humidity. Previous differential screens have identified a number of such genes based solely on their cycling expression, including clock-controlled gene 9 (*ccg-9*). Sequence analysis now shows the predicted CCG-9 polypeptide to be homologous to a novel form of trehalose synthase; as such it would catalyze the synthesis of the disaccharide trehalose, which plays an important role in protecting many cells from environmental stresses. Consistent with this, heat, glucose starvation, and osmotic stress induce *ccg-9* transcript accumulation. Surprisingly, however, a parallel role in development is suggested by the finding that inactivation of *ccg-9* results in altered conidiophore morphology and abolishes the normal circadian rhythm of asexual macroconidial development. Examination of a clock component, FRQ, in the *ccg-9*-null strain revealed normal cycling, phosphorylation, and light induction, indicating that loss of the conidiation rhythm is not due to changes in either the circadian oscillator or light input into the clock but pointing instead to a defect in circadian output. These data imply an interplay between a role of trehalose in stress protection and an apparent requirement for trehalose in clock regulation of conidiation under constant environmental conditions. This requirement can be bypassed by a daily light signal which drives a light-entrained rhythm in conidiation in the *ccg-9*-null strain; this bypass suggests that the trehalose requirement is related to clock control of development and not to the developmental process itself. Circadian control of trehalose synthase suggests a link between clock control of stress responses and that of development.

Organisms ranging from bacteria to mammals possess an endogenous mechanism that temporally organizes biochemical, cellular, and behavioral activities. These daily rhythms are produced by the circadian biological clock and are manifested by the cyclic expression of genes and gene products controlled by one or more output pathways from the clock. Circadian rhythms are coordinated with exogenous environmental cycles to limit activities to particular times of the day, thus allowing organisms to anticipate daily changes in their environment and to organize their metabolism and behavior appropriately (reviewed in reference 21).

The molecular mechanisms underlying circadian rhythmicity in *Neurospora crassa* are beginning to be understood. Components of the oscillator and input pathways of the clock have been identified and include FRQ, WC-1, and WC-2 (reviewed in references 19, 42, and 44). In addition, 12 clock-controlled genes (*ccg*s) have been identified in screens targeting output pathways (10, 43, 67), and several additional genes (*con-6*, *con-10*, *al-3*, *bli-3*, and *vvd*) are also known to be regulated by the clock (3, 29, 37, 44). Because the *N. crassa* circadian clock provides an endogenous signal to regulate asexual spore development (conidiation) on a daily basis, we initially anticipated that the *ccg*s would be associated with this developmental process. However, the levels of *ccg-7*, *ccg-8*, and *ccg-12* mRNA are not induced during conidiation, suggesting that the clock governs more than just terminal differentiation (10). For example, *ccg-7* encodes glyceraldehyde-3-phosphate dehydrogenase (59), a key enzyme in glycolysis and gluconeogenesis, and *ccg-12* encodes copper metallothionein (10), involved in metal storage and detoxification. Thus, the clock appears to regulate diverse output pathways.

Characterization of the *ccg-9* gene product now reveals extensive sequence similarity to a novel trehalose synthase (TSase) from the basidiomycete *Grifola frondosa* (56). Trehalose is accumulated by a wide variety of organisms and can be converted directly to glucose by the enzyme trehalase (33). Increased trehalose levels in fungi have been correlated with cell survival under adverse conditions, and levels of trehalose are typically high in fungal spores and stationary cultures (1, 53, 57). In *Saccharomyces cerevisiae*, for instance, trehalose is crucial for survival at high temperatures, under which conditions it functions to protect proteins and membranes from heat

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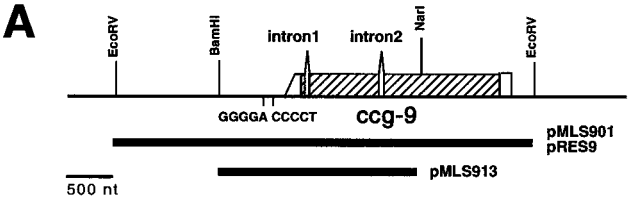

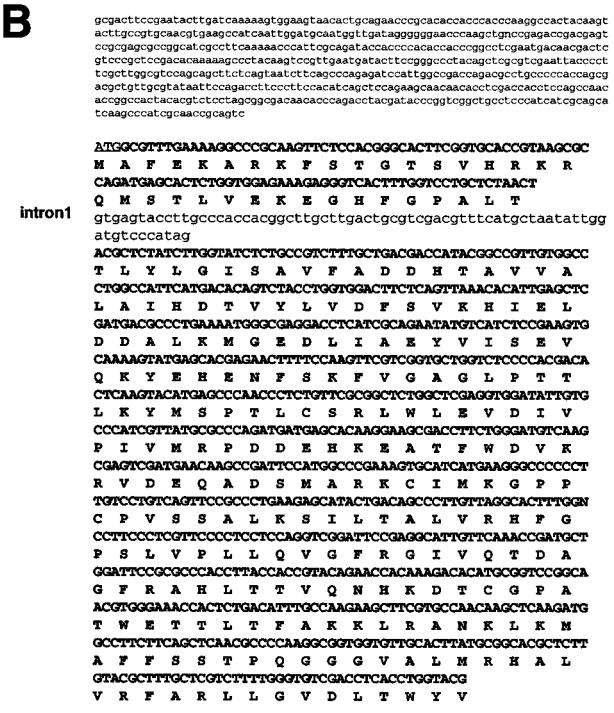

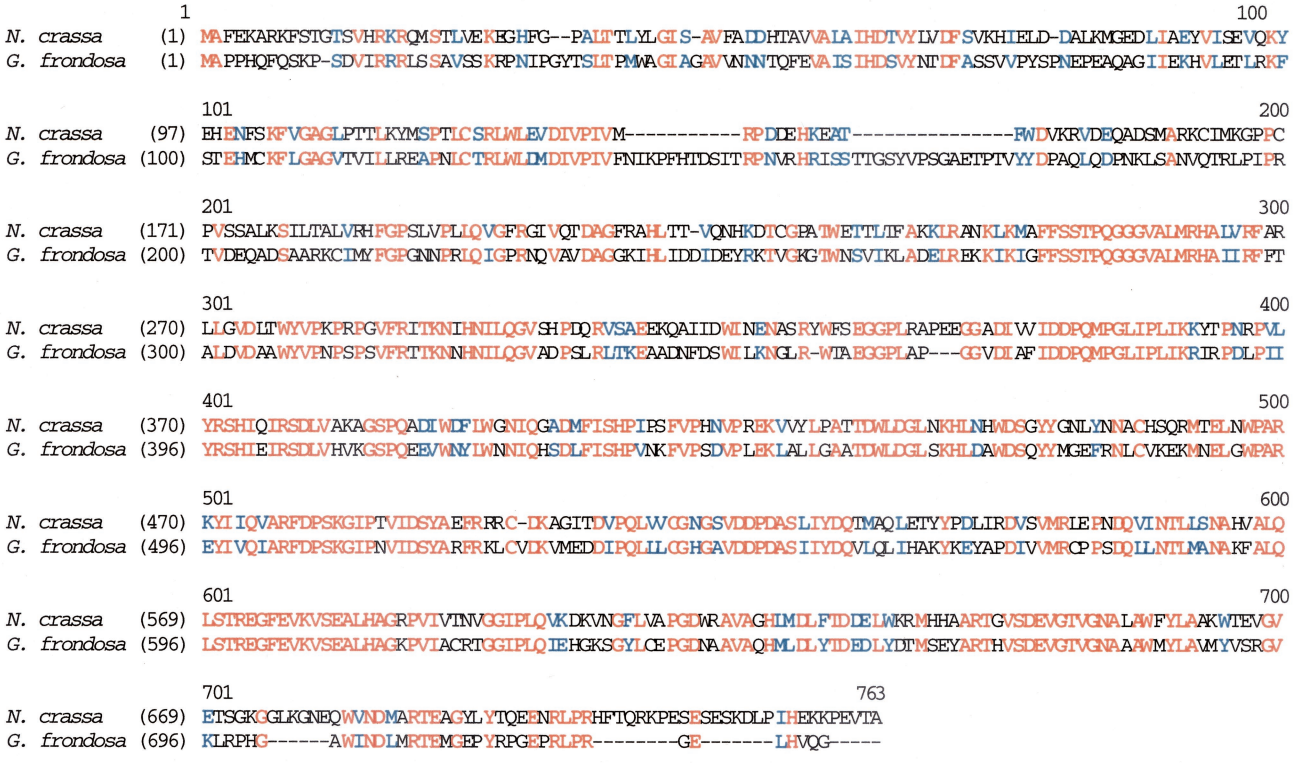

denaturation and to suppress aggregation of heat-denatured proteins (38, 60). Additionally, trehalose has recently been shown to play an important role in protecting *S. cerevisiae* from oxidative damage by free radicals (11). Accumulation of trehalose in *S. cerevisiae* is induced not only by heat but also by osmotic stress, nutrient starvation, and desiccation (6, 32, 61, 65). In *N. crassa*, a 45°C heat shock stimulates TSase activity, resulting in high levels of trehalose accumulation (47). Trehalose is also rapidly metabolized upon resumption of active growth and likely contributes energy during spore germination under conditions of limited external carbon sources (18).

In *N. crassa* and other microbes, developmental cycles are often initiated by the same environmental stresses that induce high levels of trehalose, including carbon starvation, increased temperature, and desiccation. Moreover, the process of development itself is considered to cause physiological stress on organisms. Ultimately, in dormant conidia, higher levels of trehalose and stress response proteins may be required for resistance and survival (26, 52). Thus, trehalose may play a dual role in the cell, functioning as a reserve carbohydrate and as a stress protectant.

The identification of CCG-9 as a TSase prompted an examination of its regulation and role in the cell. Here we show that expression of *ccg-9* increases during glucose deprivation and osmotic stress and, to a lesser degree, during heat treatment of developing cultures. A *ccg-9*-null mutant produces morphologically abnormal spores, suggesting that CCG-9 is involved in developmental morphogenesis of the asexual conidiospores. This function is consistent with our finding that the levels of *ccg-9* message increase when spore development is induced by desiccation (10). Strikingly, circadian regulation of asexual spore development (conidiation) and the expression of several other *ccg*s are perturbed in the *ccg-9*-null strain, despite apparent normal functioning of the circadian oscillator. This loss of normal rhythmic conidiation in the dark can, however, be circumvented by synchronizing cultures to a 12-h light/12-h dark cycle, implying that imposition of a daily light cycle eliminates the requirement of TSase activity for overt rhythmicity.

Expression of *ccg-9* mRNA peaks at the time of initiation of conidiation (10). The loss of circadian amplitude in expression for some *ccg*s in the *ccg-9* mutant strain may reflect a pleiotropic effect on the cell caused by the inability to cope with these pressures. Together these data suggest that the circadian clock, in regulating TSase expression, plays an important role in preparing cells for stress during normal spore development.

MATERIALS AND METHODS

Plasmids and culture conditions. The *ccg-9* gene was identified during a differential screen of morning versus evening cDNA libraries produced in λZapII (10); expression of *ccg-9* peaks in the late subjective night. Plasmid pCCG9 harbors *ccg-9* cDNA in pBluescript II SK(+) and was isolated by in vivo excision of the plasmid containing the cloned *ccg-9* cDNA from λZapII (Stratagene). A 4.5-kb *Eco*RV fragment of genomic *ccg-9* DNA (Fig. 1A) was subcloned into pBluescript II SK(+) to generate plasmid pMLS901. Plasmid pMLS913 was generated by cloning a *Bam*HI-*Nar*I genomic DNA fragment into pDE3 (Fig. 1) for targeting to the *his-3* locus (20). This construct was used for gene disruption by repeat-induced point mutation (RIP) (13). Plasmid pRES9 was constructed by inserting the *ccg-9* genomic DNA fragment used to make pMLS901 into pDE3. Bacterial strain XL1-Blue (Stratagene) was used for all plasmid manipulations.

Rhythmic RNA and protein analyses were carried out by using submerged liquid cultures to curtail development as previously described (25, 41, 43). Mycelial samples were grown in constant light for 4 h and then transferred to constant darkness. The light-to-dark transfer synchronizes the cells and sets the clock to circadian time (CT) 12. (Circadian time is a formalism used to compare the properties of circadian rhythms from organisms or strains with different endogenous periods, whereby the period is divided into 24 equal parts with each part defined as 1 circadian hour. By convention, CT 0 represents subjective dawn and CT 12 represents subjective dusk.) From the time of transfer to constant conditions, the clock runs with its endogenous rhythm until it is perturbed by an external stimulus. All subsequent operations were performed under a red safelight known to have no entraining effect on the clock in *Neurospora* (58). Liquid medium for the growth of cultures was 1× Vogel's salts containing 2% D-glucose, as previously described (16, 17). L-Histidine (Sigma) was added as required. Race tube medium consisted of 1× Vogel's salts, 1.7% L-arginine–HCl, 1.0% D-glucose, 0.05% L-histidine, and 1.5% agar. All *N. crassa* cultures were maintained at 25°C except during heat shock experiments.

Screening of the genomic library, sequencing, and computer analyses. The cosmid library pSV50 (Fungal Genetics Stock Center) was screened by colony hybridization with an [α-^{32}P]dCTP-labeled *ccg-9* cDNA probe to identify a genomic clone. Automated sequencing of both strands of cDNA and genomic DNA was accomplished using the Prism dideoxy sequencing kit (ABI) with nested oligodeoxynucleotide primers. DNA and putative amino acid sequences were compared to other known genes and peptides using the BLAST search of the GenBank/EMBL nonredundant database as accessed through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST). TMpred (31) and PSORT (46) were used to predict protein structure and cellular localization. Quantification of Northern and Western blots was performed on scanned images (Silverscanner III; LaCie) by densitometry, using the NIH Image 1.60 program, and linearity of the signal was confirmed by scanning blots loaded with various amounts of sample.

***N. crassa* transformation and RIP (repeat-induced point mutation).** Plasmids were introduced into *N. crassa* by electroporation with an Electroporator II (Invitrogen). pMLS913 (Fig. 1) was used to transform *his-3 a*; *bd* strain (lab strain 87–74) by targeting this construct to the *his-3* locus. To inactivate *ccg-9* by RIP (13), *his*$^+$ homokaryotic transformants were examined by Southern analysis for proper integration of the plasmid, and the resulting strain was crossed to the *his-3 A*; *bd* strain (lab strain 87–12). Mature spores from the crosses were picked and heat shocked at 60°C for an hour to induce germination. The progeny were examined by Northern analysis for lack of expression of *ccg-9*.

Environmental-stress experiments. The *bd*; *A* strain was cultured in standard Vogel's medium (17) and transferred to a modified medium to apply the desired environmental stress. Except for the heat shock experiment, all manipulations were performed in the dark, and the samples were harvested at the same developmental age (24 h after transfer to the dark) and at the same circadian time (CT 15, subjective evening). For heat shock analyses, mycelia were desiccated to induce conidiation. RNA was isolated from tissue harvested 1, 2, 4, 6, and 10 h after developmental induction at 25°C with or without a 1-h heat shock at 50°C prior to harvest, as previously described (39). Osmotic stress was applied by transferring mycelia into growth medium containing 4% NaCl and culturing for the indicated times. To examine possible effects of glucose and nitrogen deprivation on *ccg-9* expression, cultures were transferred to 1× Vogel's medium lacking glucose or 1× modified Vogel's medium (Vogel's salts lacking NH_4NO_3) containing 2% glucose. Before transfer to nutrient-deficient medium, mycelial pads were washed four times in the appropriate nitrogen- or glucose-deficient

FIG. 1. *ccg-9* gene and CCG-9 protein; *ccg-9* encodes a TSase. (A) Schematic representation of the *ccg-9* gene. Transcribed regions are indicated with boxes. Coding regions are hatched, and nontranslated regions are white. Stress response elements (GGGGA and CCCCT) are shown in the promoter region. The thick horizontal bars designate cloned regions of three *ccg-9* constructs used in this study. (B) Sequence of *ccg-9*. The coding region and corresponding amino acids are in boldface, and the sequences of the two introns are in lightface. (C) The sequence of CCG-9 shows extended similarity to a novel TSase from *G. frondosa*. Sequence alignment was generated by the ClustalW method with the AlignX program in the Vector NTI suite (InfoMax). Red and blue letters denote identical and conservative amino acids, respectively. Black letters indicate nonhomologous amino acids. Gaps are shown with hyphens. Only strong similarities are considered in consensus calculation.

medium. Genes used as positive controls were *ccg-1* for glucose deprivation and osmotic-stress experiments (40, 45) and *eas* (*ccg-2*) for nitrogen deprivation experiments (62).

Light pulse experiments. A 2-min light pulse (21 μmol of photons/m^2/s) was delivered to liquid cultures of *N. crassa* that had been held for 24 h in constant darkness (CT 15) as previously described (14, 15). Samples were harvested 15 min after the cultures had been returned to the dark.

Microscopic observation. Conidiospores were inoculated onto 1.5% agar slants containing 1× Vogel's medium, 2% sucrose, and 0.05% L-histidine with or without 3% trehalose and examined by microscopy (Olympus BH2 compound microscope) after 42 and 70 h of incubation in constant light or dark.

Race tube experiments. The circadian rhythm of developmental potential was assayed on race tubes under standard conditions (for examples, see references 22, 44, and 58). Conidia were inoculated at one end of a race tube (a 30-cm glass tube bent upward at both ends to hold an agar growth medium), and the cultures were incubated in the light for about 1 day. The growth front was then marked, and the tube was transferred to constant darkness, which sets the circadian clock to dusk; the free-running rhythm was then examined under constant conditions. The growth front was marked every 24 h thereafter under a red safelight. During vegetative growth on the agar surface, a signal from the circadian clock causes conidiation to be initiated in the late evening, beginning with the production of aerial hyphae, which eventually form restrictions to give rise to conidiospores. During subjective morning, the clock reverses the signal, and growth continues for the rest of the subjective day and into the evening as undifferentiated vegetative hyphae (42, 44). In practice, the conidiation rhythm is monitored in strains carrying the *band* (*bd*) mutation, which allows clearer visualization of the rhythm than in true wild-type strains (58; reviewed in reference 44) without affecting the underlying clock; for this reason, all strains used here contain *bd* and are referred to as clock wild type, or simply as wild type. The light-dark cycle regimens are described in Results. All race tube experiments were carried out at 25°C.

Nucleotide sequence accession number. The genomic DNA sequence of *ccg-9* was deposited in the GenBank database (AF088906).

RESULTS

***ccg-9* encodes TSase.** A *ccg-9* genomic clone was isolated from an *N. crassa* cosmid library in pSV50, and a 4.5-kb *Eco*RV fragment containing the *ccg-9* transcription unit was subcloned into pBluescript SK II(+) to generate pMLS901 (Fig. 1). Sequence analysis and comparison of the *ccg-9* genomic fragment and a *ccg-9* cDNA revealed two introns of 70 and 56 nt (Fig. 1) and a large open reading frame of 731 amino acids displaying a high degree of amino acid identity with a novel TSase from the basidiomycete *G. frondosa* (accession number AB010104) (56). The predicted start codon of CCG-9 is located 85 bp upstream from the 5′ end of the *ccg-9* cDNA clone, indicating that the cDNA is not full-length. The sequences are highly similar (98%) from amino acids 10 to 704 of CCG-9 and show 47% overall similarity (364 of 737 positions), with five gaps. Considering that the region of identity spans the entire open reading frame, we propose that *ccg-9* encodes an *N. crassa* TSase. CCG-9 does not contain a nuclear localization signal or any other known organelle-targeting signals. According to PSORT results and our examination, CCG-9 does not resemble typical transmembrane proteins, suggesting that CCG-9 is soluble, probably cytoplasmic. Stress response elements, which are positive transcriptional-control elements activated by stress conditions (54) observed in other TSase genes (18), are present in the promoter sequence of *ccg-9* (Fig. 1); we did not find the activating clock element, a *cis*-acting sequence element required for rhythmic transcription of the *ccg-2* gene (9), anywhere in the genomic *ccg-9* sequence.

Expression of *ccg-9* is altered under conditions of environmental stress. Because trehalose synthesis is often associated with stress responses and precedents exist in *S. cerevisiae* for induction of TSase by heat, osmotic stress, and nutrient starvation (32, 61, 65), we characterized *ccg-9* expression following these and another stress, glucose deprivation. *ccg-1* and *eas* (*ccg-2*), previously shown to respond to one or more of these specific treatments, were used as internal positive controls (Fig. 2) and *ccg-7*, which does not respond to these stimuli, served as the internal negative control (59) (data not shown). All three treatments resulted in TSase induction. Osmotic shock increases *ccg-9* mRNA by 60 min, although the response was not as strong as for the control *ccg-1* mRNA (Fig. 2A). Nitrogen deprivation caused a slight increase in the levels of *ccg-9* mRNA after 6 h (Fig. 2B), whereas glucose deprivation rapidly and dramatically increased *ccg-9* mRNA to high levels (Fig 2C). This increase began an hour after the transfer to glucose-deficient medium, slightly earlier than the increase in *ccg-1* mRNA. In contrast to this rapid response, a 50°C heat shock of cultures harvested at different developmental ages resulted in a small increase in *ccg-9* transcript levels only after several hours (Fig. 2D), which is not a classic heat shock response but reveals a response to heat treatment. Developmental induction of *ccg-9* mRNA was also observed 2 h after transfer to desiccating conditions (data not shown), a result consistent with previous observations (10). An alternate heat shock regimen, heating mycelia to 47°C, previously shown to induce the heat shock protein HSP30 (49), failed to elicit a response in *ccg-9* expression. Overall, however, the induction of *ccg-9* by environmental stressors, although variable, is consistent with a role for the *Neurospora* TSase in stress protection.

Inactivation of *ccg-9* by RIP results in an altered developmental phenotype. To gain more insight into the role of CCG-9 in *Neurospora*, we sought to determine the *ccg-9*-null phenotype, and to this end we employed RIP (13). During the sexual phase of the life cycle of *N. crassa*, genes that are present in two or more copies in the genome are recognized as duplicated and are mutated (by RIP) randomly but at high frequency as a result of GC-to-AT transitions (13). Because a sufficient number of transition mutations can inactivate a gene, this process provides a mechanism to generate a strain with a null mutation in a specific gene and was used to inactivate *ccg-9*. Specifically, plasmid pMLS913, which contains 0.8 kb of the promoter region of *ccg-9* and part of the coding region (Fig. 1), was introduced into *N. crassa* to generate a strain with two copies of *ccg-9*. Integration of the single plasmid copy at the *his-3* locus was verified by Southern hybridization (data not shown), and the transformant was used to fertilize a sexual cross (see Materials and Methods). The resulting spores were picked following maturation, and progeny that germinated (142 of 226 spores picked) were analyzed further.

Nine strains showed a distinct phenotype of reduced production of aerial hyphae and conidia, and these strains also grew slowly on agar slants. Different levels of morphological abnormality were observed among the progeny, suggesting the generation by RIP of an allelic series of partially functional *ccg-9* loci. To gain a broader perspective on expression levels among the progeny, Northern hybridization was performed with RNA isolated from 44 progeny, including those with altered phenotypes. One strain, MLS9352, displayed a severely altered phenotype, completely lacking detectable *ccg-9* transcripts even after prolonged exposure of the autoradiogram

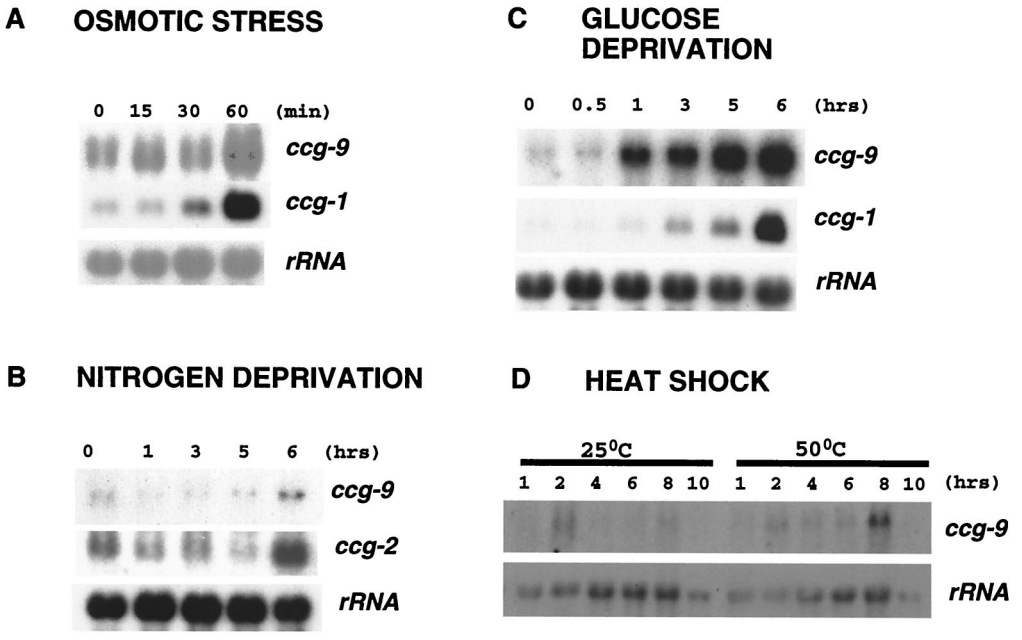

FIG. 2. Expression of *ccg-9* is affected by exogenous stimuli that induce stress. Northern analysis of *ccg-9* mRNA was performed following an environmental challenge. (A to C) Cultures were treated 24 h after transfer to darkness, a time corresponding to early subjective night (CT 15), and harvested at the indicated times after treatment. Genes affected by the different stimuli were used as internal positive controls, and 18S rRNA was used to verify equal loading of total RNA. (A) Osmotic stress induced by a change from 0 to 4% NaCl; (B) nitrogen starvation induced by a shift from 0.2 to 0% NH_4NO_3; (C) glucose starvation induced by a shift from 2 to 0% glucose; (D) heat shock induced by a shift to 50°C. For panel D, developing cultures were incubated for the indicated time at 25°C with or without 1 h at 50°C prior to harvest.

either when cultured in the dark (Fig. 3) or after light induction (data not shown). This strain was chosen for further study as an apparent *ccg-9* loss-of-function mutant.

Microscopic examination of MLS9352 cultures revealed severe defects in conidiophore development compared to wild-type cultures. In wild-type cultures grown for 40 h in the light (Fig. 4A) or the dark (data not shown), numerous conidiophores and wild-type round free conidia were detected. However, following 40 h of growth of MLS9352 in the light, only a very few, and misshapen, conidiophores were observed; no free conidia were evident (Fig. 4A). In addition, a large number of vacuoles were detected in the hyphae of MLS9352. When the cultures were incubated in the dark for 40 h, development was not evident (data not shown). As expected, following 70 h of growth in light, normal conidiophores and abundant spherical conidia were present in the wild-type strain. In contrast, MLS9352 had fewer, elongated conidia that appeared to have a reduced ability to separate into individual macroconidiophores. Similar defects were observed when MLS9352 was grown in the dark for either 70 or 96 h (data not shown).

To verify that the altered conidial phenotype results from inactivation of *ccg-9*, uninucleate microconidia were induced in MLS9352 (20) and used for transformation with a wild-type copy of *ccg-9* in pRES9 (Fig. 1) to obtain strain MLS921-10. The ectopic *ccg-9* fragment in this strain is rhythmically expressed, indicating that all the sequences required for rhythmicity are present in the fragment (data not shown). Normal development is restored in MLS921-10, as well as in MLS9352 supplemented with 0.3% trehalose, and the conidiophores appear to be normal (Fig. 4A). Furthermore, at 70 h, both the *ccg-9* rescue strain MLS921-10 and the *ccg-9^{RIP}* strain MLS9352 supplemented with trehalose produced abundant conidia with a normal spherical structure (Fig. 4B). The ability of exogenous trehalose to rescue the conidiation defect provides additional evidence that *ccg-9* encodes TSase.

Interestingly, the loss of TSase in *ccg-9^{RIP}* MLS9352 also affected growth rate. The daily growth rate of MLS9352 at 25°C in constant light decreased from the wild-type value of about 3.5 cm/day to 1.5 to 2 cm/day. Only partial restoration of the growth rate was observed in MLS921-10 (2.5 to 3 cm/day). As might be expected in cells lacking the stress protectant

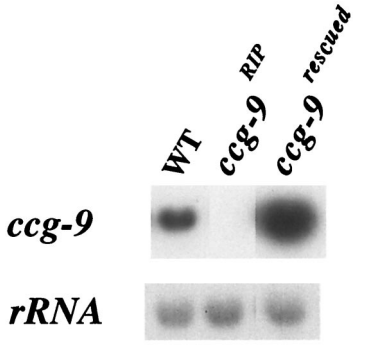

FIG. 3. Loss of transcript in *ccg-9^{RIP}* can be rescued. Shown are Northern analyses of mRNA probed with a *ccg-9*-specific probe. The *ccg-9^{RIP}* strain MLS9352 verifies loss of expression compared to a *his-3 bd* clock wild-type strain (WT), and the *ccg-9*-rescued strain MLS921-10 shows restoration of expression. Liquid cultures of mycelia were grown as described in Materials and Methods and harvested 6 h after transfer to darkness (CT 19). The blot was hybridized to rRNA for a loading control.

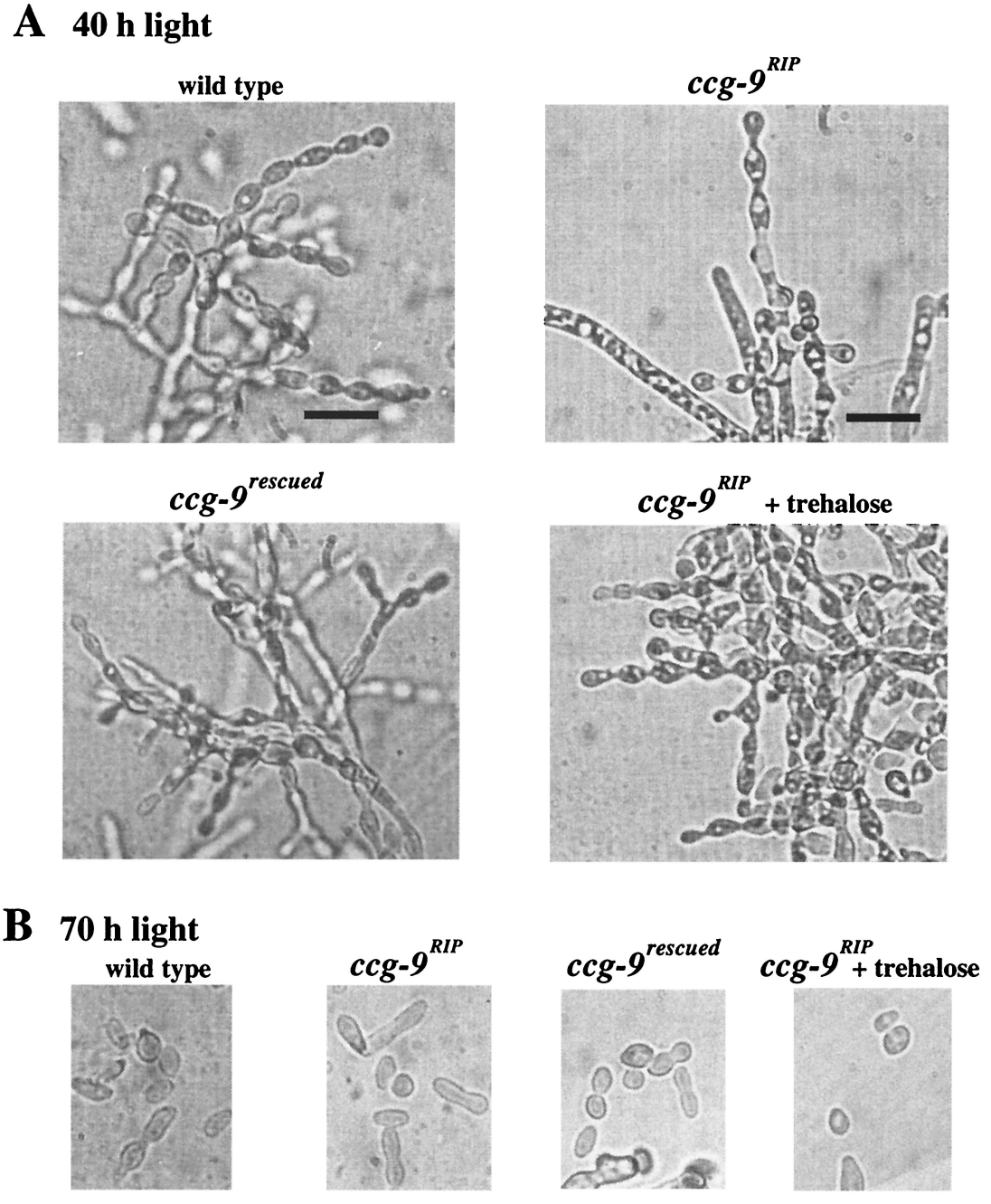

FIG. 4. Loss of *ccg-9* expression results in defects in the morphology of asexual macroconidiophores. Microscopic analysis of spores from the wild-type strain (*bd*; *A*), the *ccg-9*^{RIP} strain MLS9352, and the *ccg-9*-rescued strain MLS921-10 are shown. Also shown are spores from *ccg-9*^{RIP} cultures supplemented with 0.3% trehalose. (A) Cultures were examined after 40 h of growth in constant light. (B) Conidia were examined after 70 h of growth in constant light. Magnification, 1,000×. Bars, 10 μm.

TSase, the growth defect of MLS9352 was more pronounced at higher temperatures: when cultures were grown at 42°C in the light, the growth rates were 2.5, 1.6, and 0.3 cm/day for wild type, MLS921-10, and MLS9352, respectively. Together, these results are consistent with a role for CCG-9 in mycelial growth, conidiospore development, and in heat protection.

***ccg-9* is required for rhythmic conidiation in the dark.** Normally the circadian system of *Neurospora* dictates the times of day during which cultures are capable of entering the developmental process giving rise to aerial hyphae and conidia (44). Given the morphological defects we observed coincident with the loss of *ccg-9* transcript, we inferred that there might be an effect on circadianly regulated conidiation, and although loss of CCG-9 resulted in reduced growth and conidiation, the residual levels would still allow clock regulation of development to be assessed on race tubes (see Materials and Methods), so this was done. Surprisingly, inactivation of *ccg-9* completely abolished the overt rhythm in conidiation (Fig. 5). To

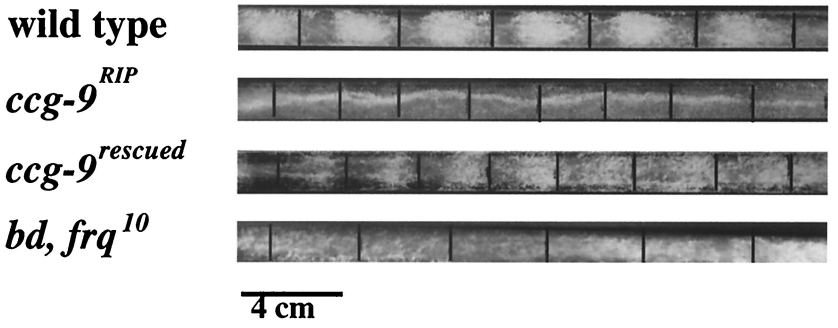

FIG. 5. Inactivation of *ccg-9* abolishes the circadian conidiation rhythm. Race tubes were inoculated with the indicated strains and the overt circadian rhythm analyzed as described in Materials and Methods. For comparison, loss of the conidiation rhythm in a *frq*-null strain is also shown. Vertical bars on the race tubes mark the growth front at 24-h intervals.

demonstrate that this effect was specific to the inactivation of *ccg-9*, the rhythm was reexamined in the rescued transformed strain MLS921-10 and shown to be restored, confirming that loss of *ccg-9* is responsible for the mutant clock phenotype. The *bd*; *A* strain (clock wild type) and the *frq*-null (frq^{10}) strain (noncircadian) (2) are shown for comparison (Fig. 5).

The observed overt arrhythmicity in the $ccg\text{-}9^{RIP}$ strain MLS9352 could arise from either a direct effect within the circadian oscillatory system itself, an inability of light to synchronize the clock (for example, see reference 14), or a defect in coupling of the oscillator to the developmental process. To determine if *ccg-9* plays a role in the control of the circadian clock by light, we examined induction of *frq* mRNA, a component of the circadian oscillator, by light in MLS9352. Previous studies demonstrated that *frq* mRNA is induced to high levels following a 2-min light pulse in wild-type strains (15). Our data show that *frq* is normally light induced in MLS9352 (Fig. 6A) and as a result indicate that *ccg-9* does not function in a light input pathway to *frq*. To distinguish between the remaining possibilities, we investigated first whether FRQ, one of the components of the *N. crassa* circadian oscillatory system (2, 8, 25, 44), is properly expressed and modified in MLS9352. Western analysis of FRQ levels demonstrated that rhythmic accumulation and phosphorylation of FRQ in MLS9352 is similar to that in wild-type strains (Fig. 6B). In five repetitions of this experiment, small differences were always observed, suggesting that FRQ may have a slightly longer period in MLS9352. FRQ always cycled and was modified in a typical time-dependent fashion similar to the wild type. In addition, wild-type rhythmic *frq* mRNA accumulation was observed in the mutant (Fig. 6C). Loss of TSase shows multiple effects on the metabolism of the organism that may include some disturbance of the circadian oscillator feedback cycle. Clearly, the FRQ-based circadian oscillator is operating in a $ccg\text{-}9^{RIP}$ strain with the major defect apparently located in the transduction of time information from the oscillator to the visible overt rhythm.

A possible explanation for the loss of the conidiation rhythm might be that CCG-9 is required to complete an output signal that is initiated by the oscillator to regulate conidiation. We examined accumulation of known *ccg* mRNAs over the course of 2 days in MLS9352. Northern blot assays showed that rhythmic mRNA accumulation was observed for most of the *ccg*s, although the amplitudes were often lower than that seen in wild-type cells. Robust circadian rhythmicity was not observed for *ccg-4*, *ccg-7*, and *ccg-12*, and the amplitude of the rhythms was consistently altered in *ccg-6* (Fig. 6C). However, after repetitions of these experiments, it was difficult to determine if disruption of *ccg-9* abolishes or simply diminishes rhythmicity of these genes. Although we cannot rule out the possibility that *ccg-9* has a role in signaling time-of-day information to other *ccg*s, this seems unlikely, since no role in transcriptional regulation has previously been attributed to TSase. It is more probable that the effects on *ccg* expression are the result of the growth and developmental defects in the mutant and are indirect.

The circadian rhythm of development in *N. crassa* normally persists under constant environmental conditions with a periodicity of approximately 22 h. This rhythm can be synchronized or entrained to precisely 24 h using a periodic 12-h light/12-h dark cycle (12, 35). To investigate whether the conidiation defect in strain MLS9352 is itself responsible for the loss of rhythmicity in cultures grown under constant-dark conditions, we examined the ability of MLS9352 to form conidial bands on race tubes when the clock is entrained to light-dark cycles of 12 h each. Both the *bd*; *A* strain and MLS9352 produced clear bands of conidia, with conidiation initiating every 24 h at the end of the dark period (the late evening) and ceasing after a short time in the light (in early morning) (Fig. 7). In frq^{10} cells lacking the clock component FRQ, the clock-regulated developmental rhythm was not restored by the light-dark cycle (Fig. 7). As expected, in the absence of the clock, somewhat denser asexual development can be seen during the light phase, as development is a light-inducible event independent of the clock. This result suggests that the developmental defect in MLS9352 does not preclude rhythmic conidiation and further confirms that the oscillator functions normally in the $ccg\text{-}9^{RIP}$ mutant. Thus, despite the finding that the conidiation defect in the *ccg-9*-null strain is similar in light- and dark-grown cultures, these data indicate that a daily light treatment can bypass the requirement of TSase for expression of conidiation rhythms.

DISCUSSION

The *ccg-9* gene, whose mRNA accumulates to peak levels during the late night, is one of six circadianly regulated genes identified during a systematic screen for genes involved in circadian output in *N. crassa* (10). Here we have shown that *ccg-9* encodes a TSase, a member of a class of proteins normally associated with cellular stress responses. Consistent with

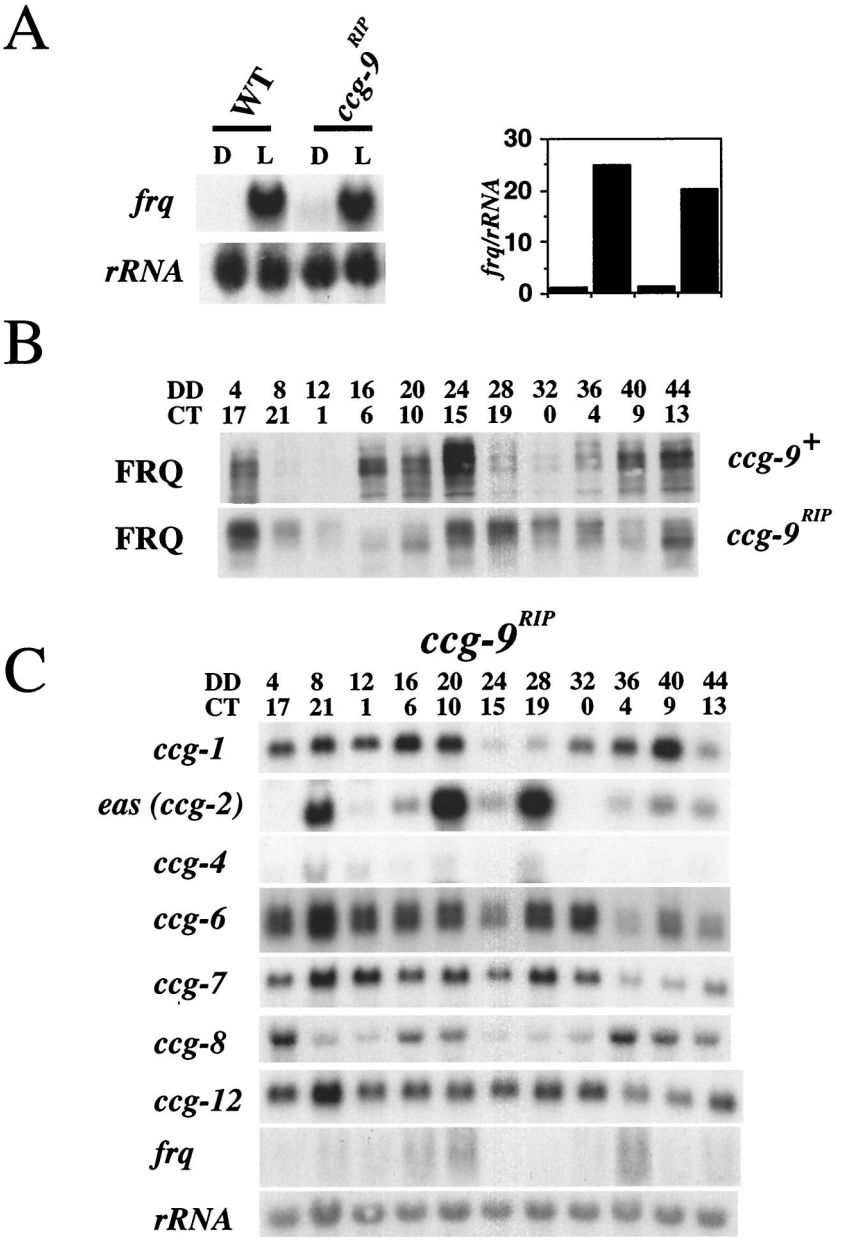

FIG. 6. Light input and the circadian oscillator are operational in a loss-of-*ccg-9* strain. (A) Light induces *frq* mRNA to normal levels in the *ccg-9*^{RIP} strain. RNA isolated from cultures with (L) or without (D) a light pulse is shown hybridized to a *frq* riboprobe. rRNA was used as a loading control, and results of a normalized densitometric analysis are shown on the right. (B) Western analysis of FRQ in the *ccg-9*^{RIP} strain shows rhythmic expression and normal modification of the clock component FRQ. Rhythmicity of FRQ levels and different phosphorylation states of FRQ, as evidenced by the more slowly migrating bands (25), are observed in both *ccg-9*-expressing (*his-3* A; *bd*) and *ccg-9*^{RIP} strains. Equal loading of protein was verified by staining the membrane with amido black (not shown). (C) Accumulation of mRNA from the indicated *ccg*s and *frq* genes over 2 circadian days in the *ccg-9*^{RIP} strain indicates that aspects of circadian output are disturbed or eliminated by loss of CCG-9 for some genes (e.g., *ccg-2*) but not for others (e.g., *ccg-1*). Northern blots were hybridized with the indicated *ccg* or *frq* riboprobe. DD, hours in the dark. rRNA was used as a loading control.

this, *ccg-9* is strongly induced by several typical stress agents, including osmotic stress and nutrient deprivation. Somewhat surprisingly, *ccg-9*^{RIP} mutant strains display developmental defects, and these can be rescued by the addition of exogenous trehalose consistent with the imputed identity of this gene. Most remarkably, the TSase encoded by *ccg-9* appears to be required for normal expression of the circadian rhythm in development in *Neurospora*, a finding suggesting a broader role for this enzyme and its product in the cell than might have been previously expected.

The identification of *ccg-9* represents the second occurrence of the novel form of TSase originally identified in *Grifola* (56). There, it was originally identified in a screen to identify a TSase capable of producing trehalose by condensation of α-D-glucose 1-phosphate from sucrose generated in the presence of sucrose phosphorylase (55). Other types of TSases are also

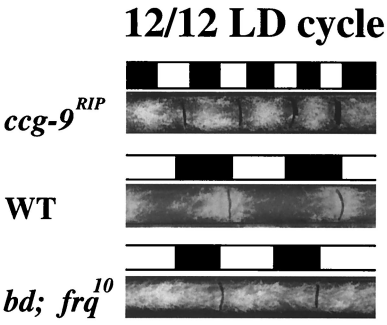

FIG. 7. Light/dark cycles entrain the conidiation rhythm in a *ccg-9^{RIP}* strain and restore the normal clock-controlled conidial banding pattern. Race tubes inoculated with the indicated strains were maintained in a 12-h light/12-h dark (LD) cycle. Black and white boxes above race tubes indicate the duration of the dark and light periods. Circadianly controlled conidiation is initiated during the dark phase of the cycle. The lower growth rate of MLS9352 is evident in the light/dark cycles. In the circadian loss-of-function *bd*; *frq^{10}* mutation, conidiation is slightly heavier when induced by light.

known; for example, in *S. cerevisiae* the products of at least three genes combine to form trehalose in a two-step process (7, 50, 61) also shared by other yeasts (24) in which UDP-glucose is first linked (via trehalose-6-phosphate synthase) to glucose-6-phosphate to generate trehalose-6-phosphate, from which phosphate removal by trehalose phosphatase yields trehalose.

The expression pattern of *ccg-9* is consistent with the observation that trehalose accumulates in cells when energy storage is beneficial, including in spores and stationary-phase cultures (61), or when stress is anticipated or encountered. First, levels of *ccg-9* mRNA increase about 2 h after conidiation is induced (Fig. 3) (10), and *ccg-9* is required for conidia to develop with a normal morphology (Fig. 4). Furthermore, expression of *ccg-9* is induced by osmotic stress or glucose deprivation (Fig. 2A and C). Surprisingly, we observed only a small induction of *ccg-9* following a 50°C heat shock in developing cultures (Fig. 2D) and several other culture regimens failed to elicit any induction of *ccg-9* transcripts following heat shock. However, previous studies with *N. crassa* demonstrated that even a 45°C heat shock results in increased TSase activity and increased trehalose accumulation (47), suggesting that the major increase in TSase activity may be the result of posttranscriptional regulation. Although it is likely that some of the stresses applied in these experiments were sufficient to reset the circadian clock and thereby, independently, result in eventual changes in the levels of *ccg-9* mRNA, it seems most probable that the effects on mRNA levels that we observed are not due primarily to clock effects. If the *ccg-9* transcript accumulated because the clock was phase shifted to a new phase during which *ccg-9* expression is triggered, we might expect the induction patterns of the transcripts of the *ccg-1* and *ccg-2* controls to be similar, since clock-controlled expression of these genes peaks at approximately the same time of day (10); this was not seen. Also, given the rapid kinetics of the induction, it is unlikely that enough time would be available for the stresses to exert their effect by acting first on the oscillator and thereby secondarily on *ccg-9*.

The *ccg-9* gene product does not function directly in the oscillator or within the light input pathway to the oscillator. This conclusion is based on the observation of normal oscillations in *frq* mRNA, FRQ protein, and FRQ phosphorylation (Fig. 6A and C) and the normal induction of *frq* mRNA by light in the *ccg-9*-null strain (Fig. 6B). Given this, it was particularly interesting to find that the conidiation rhythm in constant darkness was abolished in the mutant (Fig. 5). The amplitudes of the rhythms in mRNA accumulation for some, but not all, of other known *ccg*s were affected in cells lacking CCG-9 (Fig. 6C). These results suggest either that CCG-9 directly affects the expression of other *ccg*s within an output pathway from the clock or that the loss of TSase has generalized pleiotropic effects on the cell. Since no precedent exists for a TSase directly regulating gene expression, we suggest that reduced stress protection during conidial development results in secondary effects on gene expression in the mutant. Pleiotropic effects from inactivation of TSase are also observed in *S. cerevisiae* (63), *Schizosaccharomyces pombe* (53), and *Candida albicans* (66).

Surprisingly, a robust light/dark cycle was found to restore conidial banding with a wild-type phase in the *ccg-9^{RIP}* mutant (Fig. 7), demonstrating that the altered conidial morphology in the *ccg-9^{RIP}* mutant is not the cause of the observed arrhythmicity in constant dark conditions. In *frq^{10}* cells that lack normal oscillator function, rhythms were not restored by a light-dark cycle. Thus, the light signal still needs to be processed through the clock to observe circadianly rhythmic conidial banding. At present, we do not understand why conidial banding is absent in *ccg-9^{RIP}* mutant cultures grown in the dark but is present in those grown in light-dark cycles, particularly since the defect in conidiation is observed in both light- and dark-grown cultures. One possibility is that robust cycling of some of the output *ccg*s is required for the conidiation rhythm and that the decrease in the amplitude of circadian fluctuations in the expression of some of the *ccg*s elicited by loss of CCG-9 may be overcome by a daily light cycle. In this scenario, the daily light treatment might induce gene expression and mimic the circadian pattern of *ccg* expression normally seen under constant dark conditions. Consistent with this possibility, many of the *ccg*s, especially those whose function is thought to be linked to development, are photoinducible (4, 5, 10, 36).

Like *ccg-9*, two other *ccg*s, *ccg-1* and *con-10*, are induced during conidiation and regulated by various stresses, including heat shock and carbon starvation (39, 40, 45), suggesting that the products of these clock-regulated genes may function as stress response proteins; data have also suggested that HSP70 is under circadian control (reviewed in reference 51). In other fungi, the production of stress response proteins also increases during sporulation (30, 34, 64), and in *N. crassa*, the highest levels of the stress-response proteins GRP78 and HSP70, which are involved in the transport, folding, and assembly of newly synthesized proteins, are observed in conidiating aerial hyphae and in dormant conidia (23, 26, 52). Thus, a high concentration of trehalose and large amounts of stress-response proteins likely render conidiospores resistant to a variety of environmental stresses. In addition, these proteins may be important for the correct folding of newly synthesized proteins and might play a role in the expression of new proteins required during conidial development and subsequent germination (26, 60). Recently, circadianly regulated transcripts for three trehalose 6-phosphate synthase isoforms were isolated in

an oligonucleotide microarray analysis using *Arabidopsis* (27). The finding that TSase and other stress-related proteins are regulated by the clock strongly argues for an important role of the circadian clock in anticipating and preparing for daily stress, including that encountered during conidial development. Consistent with this idea, the *ccg-9* transcript peaks in accumulation in the late evening, CT 19, the same time that conidiation is initiated by the circadian clock (10, 44). This suggests that the clock regulates the expression of stress response proteins in anticipation of the physiological stress imposed by the conidiation event, rather than accumulation occurring as a consequence of development.

Circadian rhythms in heat shock proteins have been observed in the cyanobacterium *Synechocystis* and in *Neurospora*, and circadian changes in thermotolerance have been documented in fungi and several plant species (reviewed in reference 51). In mammals, the levels of heat shock proteins are typically low in differentiated cells and are induced during the formation of gametes and at certain stages of embryogenesis (28). Furthermore, in mammals stress can alter circadian rhythms in control of body temperature, appetite, and locomotor activity rhythms. Repeated stress elevates the levels of plasma corticosterone in rats in the morning (48) and reduces the normal circadian amplitude. Taken together, these results and ours suggest that the circadian clock plays a central role in controlling stress responses in phylogenetically diverse organisms.

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